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16. ABSTRACT

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ULTRAVIOLET RADIATION-INDUCED NEOPLASTIC TRANSFORMATION OF NORMAL HUMAN CELLS, IN VITRO*

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SUMMARY

Human foreskin cell cultures in scheduled DNA synthesis (S phase) of the cell cycle were exposed to UV irradiation at a dose of 10 $J \cdot m^{-2}$ in the presence of insulin. These treated cell populations, when selectively passaged in a high amino acid supplemented complete growth medium (CM) after 20 Dulbecco's phosphate buffered saline (pH 6.8) (PDL), were able to be grown in soft agar. These treated cell populations were also grown in 1% serum supplemented growth medium and at 41°C in 10% serum supplemented growth medium. Cell populations 4—5 PDL after treatment exhibited altered colony morphology and altered lectin agglutination profiles but would not grow in soft agar. These events appeared to be associated with the early stages in the expression phase of the transformed phenotype. After 20 PDL, we observed that these cells would grow in soft agar at a frequency of 20 colonies/10^5 cells seeded in soft agar. The cell populations derived from these colonies, when propagated and injected into the nude mice, formed myxofibromas at the injection sites rather than the type of tumor (fibrosarcoma) previously described for chemical carcinogen-induced neoplasms.

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Abbreviations: CM, complete growth medium; FBS, fetal bovine serum; MEM, minimum essential medium; NFS, neonatal foreskins; PDL, Dulbecco's phosphate buffered saline (pH 6.8); RCE, relative colony-forming efficiency; S phase, scheduled DNA synthesis.

INTRODUCTION

Carcinogen-induced transformation of human cells in vitro by chemicals or irradiation has been difficult. Recently, however, neoplastic transformation of human cells by chemicals [1] and viruses [2] has been achieved. In addition, Sutherland [3], using UV treatment of human cells, damaged the DNA and transformed the cell populations to an anchorage independent state of growth. Multiple treatments at subtoxic doses of UV were necessary to damage the DNA and induce transformation. Other studies with refractory human cells have indicated that carcinogens can induce unscheduled DNA synthesis [4] or repair synthesis [5]. However, these reports do not attempt to correlate damage to DNA with expression of carcinogenesis. Instead, Heflich [6] correlated removal of chemical carcinogen and induced damage to DNA with cytotoxicity. We have expanded on our preliminary report of UV-induced neoplastic transformation of human cells [7] and present data on the reproducibility of the process. We also present data on the interrelationship between anchorage-independent growth and tumor growth as the normal cells pass through a retro-differentiated sequence from induction to neoplasia.

MATERIALS AND METHODS

In order to have observed reproducible transformation of human cells exposed to UV, it was necessary, first, to use low passaged cell populations (PDL 1—5) and, second, to complete a cell survival curve prior to the selection of an appropriate treatment dose.

Cell cultures

Primary cell cultures were obtained from neonatal foreskins (NFS) as previously described [8]. They were maintained on CM, viz., minimum essential medium (MEM): 25 mM Hepes, Gibco, Grand Island, New York) at pH 7.2, supplemented with 10% fetal bovine serum (FBS), (Rehatuin, Kankakee, IL), 1 mM sodium pyruvate, 2 mM glutamine, 0.2% sodium bicarbonate and 50 μg/ml gentomycin, in an atmosphere of 4.0% CO_2-enriched air at 37°C.

Survival studies of irradiated cells

Immediately after UV treatment or 40 h later, cell survival was measured by either dye exclusion [9], colony-forming ability [10] or incorporation of [^{3}H]thymidine into cellular DNA [9]. After seeding, cultures were irradiated with a 15 W Germicidal Electric lamp (15GT8) at a fluence rate of 1.2 $J \cdot m^{-2} \cdot sec^{-1}$. The fluence rate was measured by a Blak-Ray UV meter (UV Products, International, San Gabriel, CA). To measure colony forming capability of the treated cell population, one thousand cells were seeded in 25 cm^2 wells (Falcon Plastics, Oxnard, CA) and fed with CM supplemented with 20% FBS. These cultures were incubated at 37°C in a

4% CO_2-enriched air atmosphere for 9 days, fixed in phosphate-3% formalin stained with hematoxylin-eosin and enumerated under 23×. If the effect of the treatment on cell survival was to be measured by dye exclusion, approx. 5000 cells · cm^{-2} in a 25-cm^2 dish were trypsinized at the conclusion of the UV treatment and counted in a hemocytometer [9]. Inhibition of the incorporation of CH_3-[^{3}H]thymidine (S.A. 54.0 Ci/mmol) into the DNA of UV-treated cells was measured by sampling the cell population from 0—48 h following initiation of UV treatment [8]. At 1-h intervals, 3 coverslip cultures were removed, fixed in 3 : 1 methanol/acetic acid solution, acid washed in 1.0 N HCl and air dried. Two coverslips were transferred to scintillation vials and incubated overnight with 0.5 ml of NCS tissue solubilizer, then neutralized with 1 N NaOH. Insta-Gel (10 ml) (Beechman Inst., Palo Alto, CA) was added to each vial and the radioactivity counted in a Beckman scintillation counter.

Induction

The scheme of induction and selection of the transformed cells will be presented along with the chronology of the events as they occurred (Fig. 1).

An irradiation dose permitting 50% of the cells to survive was optimal for the formation of transformants in each cell population studied [1]. Preconfluent, logarithmically growing cultures at low (3—8) PDL were synchronized by placing them at a density of 5000 cells · cm^{-2} into Dulbecco's Modified Eagle's Medium (Biolabs, Northbrook, IL) at pH 7.2, supplemented with 10% dialyzed FBS but lacking arginine and glutamine. After 24 h, the amino acid deficient medium was replaced with CM containing 0.5 U/ml of insulin. Then hours later, when the cell population was entering S phase of the cell cycle [1], the growth medium was removed, the cultures were washed twice with PDL, irradiated with 5.0 J · m^{-2} of UV and refed with CM containing 0.5 U/ml insulin. Upon completion of the treatment, the cells were allowed to proceed through S phase, which required 8.2 h, and one mitosis. They were then split 1 : 2, then 1 : 10, into MEM supplemented with 10% FBS plus 8× non-essential amino acids (Microbiological Assoc., Rockville, MD) (8× growth medium). The 8× growth medium inhibited growth of cells that exhibited a normal phenotype [1] while allowing proliferation of treated-altered cells.

The treated cell population at this time was identified as entering the early stage of the carcinogenesis process. During this stage of expression it is imperative that no part of culture remain in a saturation density growth state for more than 16 h prior to passaging the culture.

Early

Growth of cells at 41°C and in medium with reduced serum concentration. Cell populations, following treatment with UV, were seeded (5000 cm^{-2}) in CM and serially passaged at 1 : 4 on a 4-day schedule and maintained at 41°C in a 4% CO_2-enriched air environment. Cell populations from companion cultures were seeded at 5000 cells · cm^{-2} in CM supplemented with

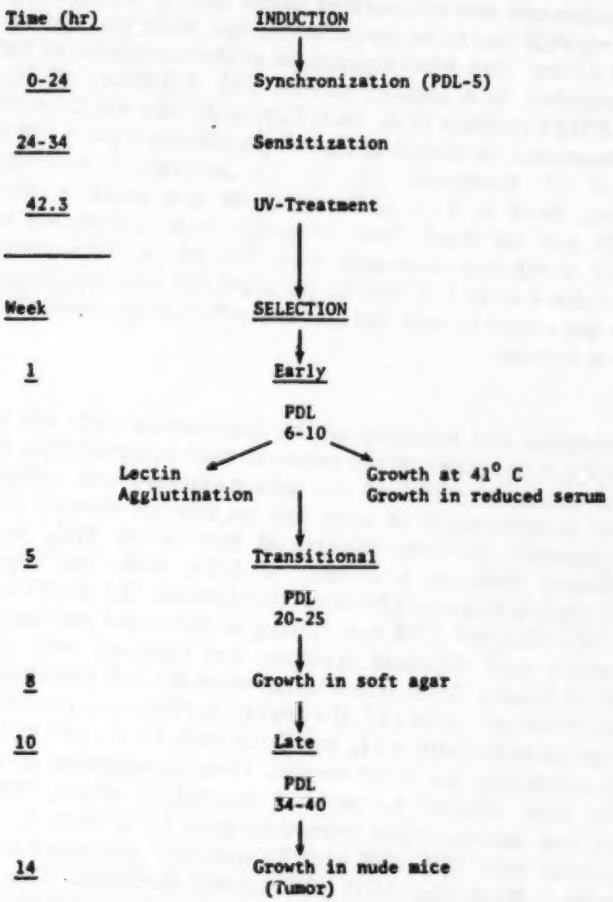

Fig. 1. The scheme followed here is a graphic representation of the sequence originating with the induction (treatment in early S) through tumor formation. Each critical stage is represented by the PDL (population doubling) reached at each stage of the process. Transition from colony formation is soft agar prior to growth in the nude mouse was achieved following isolation and seeding of colonies in 75 cm^2 flasks to produce 5×10^4 cells of inoculum for each mouse.

1% FBS and maintained also in a 4% CO_2-enriched air environment at 37°C.

Lectin agglutination. Treated and controlled populations at a 70—90% confluent density of proliferating cells were removed from the substratum of the flask by the action of 0.05% trypsin [8]. Following centrifugation at $650 \times g$, the pellet was resuspended in PBS (pH 6.8) and recentrifuged twice. The final cell suspension was left on wet ice at a cell density of 10^6 cells $\cdot$ ml^{-1}. Wheat germ agglutinin prepared in PBS was added to the

wells of microtiter plates (0.025 ml). To this, 0.025 ml of a suspension of 180 000 cells in PBS was added to each well and incubated at 21°C for 10 min. The wells were then examined immediately.

Altered colony formation. Fibroblast cultures were treated as described in the induction protocol. Four PDL, following irradiation of the treated cells, were seeded into 60-mm diameter wells at a 1000 cells/well into 8X growth medium. After incubation at 37°C in a 4% CO_2 humidified enriched air atmosphere for 9 days, the cells were fixed with 3% PBS buffered formalin (pH 7.2), stained with hematoxylin and examined for colonies with abnormal colony morphology. The abnormal colonies were comprised of polygonal-small cells that overlapped into a criss-cross disoriented growth pattern. This growth pattern was in contrast to the normal large fusiform shaped fibroblasts growing in a parallel-whorling orientation.

Transitional

Soft agar. After serial passage of the UV-treated cells for 20 PDL in 8X amino acid supplemented CM, 50 000 cells were seeded in 2 ml of 0.33% agar supplemented with modified Dulbecco'c Lo-Cal medium (Biolabs, Northbrook, IL). These cells were then overlaid on a 2 ml 2% agar base supplemented with RPMI 1629 growth medium enriched with 20% FBS. Colonies formed after 14—17 days of incubation in a 4% CO_2-enriched air environment at 37°C [1]. These cultures were refed every 7 days with 0.5 ml of Lo-Cal medium-supplemented agar. Since a close correlation has been shown between the growth of chemically transformed human cells in soft agar and their neoplastic potential [1], growth of the UV-treated cells in 6-week-old male athymic nu/nu mice from a BABL/c background (Sprague—Dawley, Madison, WI) were chosen as a suitable assay system to determine the neoplastic potential of the culture derived from UV-treated cells.

Late

Growth in nude mouse. Cells were harvested by trypsinization and resuspended in Dulbecco's EMB LoCal (Biolabs, Northbrook, IL), supplemented with 20% FBS and 1X essential amino acids and 0.35% agar. Six-week-old nude mice, which have been irradiated 3—4 days previously with 450 rad ^{137}Cs-rays, were injected subcutaneously with a cell mass of 0.5×10^7 UV-treated cells. Nodules became evident at the site of inoculation 14—21 days following injection and continued increasing in size up to 4—6 weeks. The blebs formed at the injection site regressed in 48—72 h. The tumors were excised between 4 and 6 weeks and submitted for histopathological examination.

RESULTS

Toxic response

In the early stages of our experiments following the treatment with UV,

we measured the toxic effect the treatment had on the UV-insulin treated cells by counting the total number of viable cells at 12 h (Fig. 2) after initiating the UV treatment or at 40 h after initiating treatment (Fig. 3). In addition, recovery of cell division from the toxic effects of irradiation, monitored by [^{3}H]thymidine incorporation into DNA and by cell counts, began at 44—48 h following the completion of the treatment. The response patterns of irradiated cultures previously not exposed to insulin exhibited a typical shoulder on the survival curve followed by a logarithmic decline at doses greater than 10 J · m^{-2}. However, cell cultures pretreated with 05 U/ml insulin and then irradiated lacked a shoulder at 10 J · m^{-2} and were biphasic in response at higher doses of UV. The toxic effect of increasing UV doses was measured more sensitively by relative cloning efficiency (RCE) than by the trypan blue dye exclusion technique; it took roughly a 10-fold higher dose to demonstrate a measured effect (Fig. 2) by dye exclusion than by inhibition of colony formation (Fig. 3). The slopes of each of the survival curves were similar (Figs. 2 and 3). The dose-

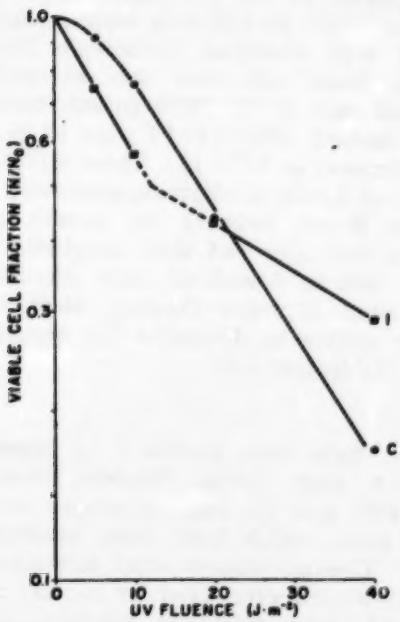

Fig. 2. Low PDL of NFS cells were seeded at 5000 cells · cm^{-2} into 60 mm diameter, 4-well plastic petri dishes and synchronized by amino acid deprivation. Following UV irradiation at a fluence rate of 1.2 J · m^{-2} · sec^{-1}, the plates were incubated at 37°C in a 4% CO_2 humidified air atmosphere. At this time cell suspensions were prepared by trypsinization and counted by trypan blue dye exclusion in a hemocytometer. The closed circles represent the irradiated cultures not exposed to insulin and the closed squares represent the irradiated cultures treated with 0.5 U/ml of insulin. Data points are the mean values for an *n* of 8.

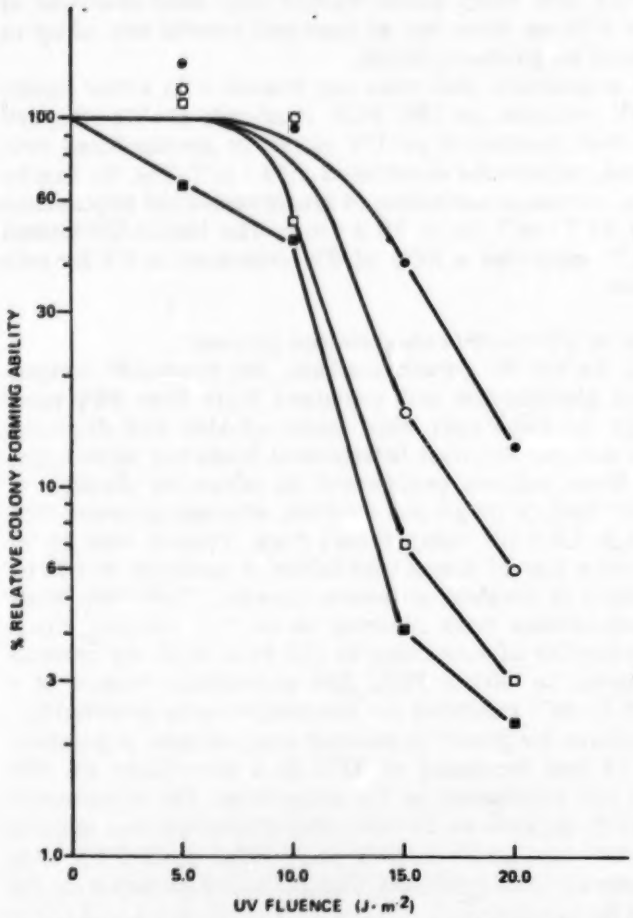

Fig. 3. Cells were seeded at 5000-cm^{-2} into a 60 mm diameter 4-well plastic petri dish containing Dulbecco's Modified Eagle's Medium supplemented with dialyzed FBS, minus arginine and glutamine (pH 7.2). These cells were released from the block (see text) and treated during the S phase with UV. Once the treated cells had completed S (8.2 h) they were seeded at a concentration of 200 cells/25 cm^2 well and cloned [3] for 11 days. The control (untreated) cultures exhibited 18—24% cloning efficiencies; results are reported as a percentage of control values. The circles (○——●) represent cells treated with UV. The squares (□——■) represent cells treated with UV and 0.5 U of insulin. The open circles and squares represent time points 12 h posttreatment at the conclusion of the S + G_2 + M part of the cell cycle, while the closed circles and squares represent time points coincident with the time points used for the trypan blue experiments (Fig. 1). Each point represents data from 10 different wells.

response profiles of cultures not treated with insulin were similar to those reported by Lehman et al. [11] or Maher et al. [12] for human skin fibroblasts. We noticed that when insulin-treated cells were irradiated at 5 $J \cdot m^{-2}$ (Fig. 3) in S phase there was an increased survival rate of up to 120% over the irradiated insulin-free cultures.

Synchronized cell populations that were not treated with either insulin or irradiated with UV exhibited an 18% RCE. Randomly proliferating cell populations treated with insulin-UV or UV alone, or synchronized cells treated with UV alone, exhibited a shoulder at 10 $J \cdot m^{-2}$ (Fig. 3). Insulin treatment appeared to increase sensitization of synchronized cell populations to UV treatment at 10 $J \cdot m^{-2}$ up to 20 $J \cdot m^{-2}$. The insulin-UV-treated cultures at 20 $J \cdot m^{-2}$ exhibited a RCE of 3%, compared to 6% for cells not treated with insulin.

Growth characteristics of UV-treated cells (selective process)

After 10–15 PDL in the 8× growth medium, the irradiated cultures (10 $J \cdot m^{-2}$) appeared pleomorphic and contained more than 95% small polygonal cells. Many of these cells were multinucleated and displayed multiple processes as seen by Contrast Interference Nomarski microscopy. Cells passaged from these cultures proliferated to saturation densities of $4–6 \times 10^6$ cells/75 cm^2 flask in 8× growth medium, whereas untreated NFS cells only reached $1.2–1.5 \times 10^6$ cells/75 cm^2 flask. Treated cells (5.0–13.0 $J \cdot m^{-2}$) exhibited a loss of contact inhibition, a tendency to pile up in culture and a pattern of irregular criss-cross growth. (Table III). None of these altered characteristics were observed in control cultures. These cell populations were capable of subpassage to 120 PDL while the controls phased out after passage to 40 ± 5 PDL. Cell populations treated at a subtoxic dose of 1.2 $J \cdot m^{-2}$ exhibited no abnormal colony morphology.

To examine the cultures for growth at elevated temperatures, populations were subpassaged 1 : 4 and incubated at 37°C in a humidified 4% CO_2 atmosphere to allow cell attachment to the substratum. The temperature was then raised to 41°C. As early as 10 PDL after irradiation, cell cultures derived from UV-treated populations actively proliferated at 41°C for 72 h, at which time they were 60–70% confluent. Control cells detached from the surface in less than 24 h.

Growth in reduced serum

Many transformed mammalian cells have been reported to grow in reduced serum concentrations [13–15]. We examined our control and UV-treated human cell populations for this characteristic after 10 PDL by subpassing them into MEM supplemented with 1% FBS. Table I represents the growth characteristics of these cultures at reduced serum levels. Control NFS cells ceased proliferating after 5 PDL. After an initially slow growth rate, the UV-treated cultures appeared to adjust to the low serum concentration, as shown by the reduction in time needed to attain confluency. They continued to replicate at least through 17 PDL in 1% FBS then were transferred back to MEM ± 10% FBS.

TABLE I

GROWTH OF CONTROL (NFS) AND UV-TRANSFORMED HUMAN CELLS AT REDUCED SERUM CONCENTRATION

NFS cells (PDL 14) and UV-treated (PDL 17) passages were transformed into MEM supplemented with 1% FBS and cellular proliferation was monitored. The NFS cultures ceased dividing after 10 PDL; however, the cells from UV-treated culture appeared to adapt to the low serum concentration.

Split ratio		Days to confluency		PDL	
NFS	UV	NFS	UV	NFS	UV
1 : 10	1 : 10	8	8	3.3	3.3
1 : 10	1 : 10	32	15	3.3	3.3
1 : 4	1 : 4	9	8	2	2
1 : 4	1 : 4	8	8	2	2
1 : 2	1 : 4	Dead	8	—	2
	1 : 4		7		2
	1 : 4		5		2
Totals				10.6	10.6

Agglutination properties of UV-treated cells

After at least 10 PDL in 8× growth medium, control and UV-treated cells were tested for agglutinability by exposure to varying concentrations of wheat germ lectin. UV-treated cells were agglutinated by 78 μg/ml of lectin, whereas control cultures of NFS cells required 2500 μg/ml, more than 30× as much [16], as the UV-treated cells.

Abnormal colony formation and growth in soft agar

Transformed cells require growth in culture before they will grow in soft agar. UV-treated cell populations were serially passaged prior to seeding in soft agar. This was in contrast to serially passaged chemical carcinogen treated cells 16—20 PDL [1]. When UV-treated cells were serially passaged through 0.35% soft agar, the average colony frequency was 20 colonies/10^5 seeded cells. None of the untreated cells would grow in soft agar. Occasionally we observed small colonies of 2—6 cells in size, but they would not produce colonies 50 cells or larger and were not viable when isolated from soft agar after a 14-day incubation period in soft agar (Table II). Of 20 different cultures treated with $UV_{254\,nm}$ irradiation, we transformed 18 of them; colony formation in soft agar was a measurement of transformation. The other 2 were refractory to the treatment. We therefore examined the cultures for variations in response to UV irradiated as a function of their ability to exhibit anchorage independent growth. We correlated this parameter with the frequency of abnormal colony formation and found that 5 $J \cdot m^{-2}$ induced the formation of a higher proportion of colonies (transitional transformants) to grow in soft-agar than 10 $J \cdot m^{-2}$, while 10 $J \cdot m^{-2}$

TABLE II

FREQUENCY OF COLONY GROWTH IN 0.33% AGAR OVERLAY OVER A 2.0% AGAR BASE

The data presented here is an average value for colony formation for 8 wells. Each well was seeded with 50 000 cells at PDL 20 into 0.33% agar over a 2.0% agar base. The colonies were counted after 21 days and the frequency of colony growth normalized to 100 000 cells per well.

Exp. No.	PDL	Growth in soft agar
1	20	20.0
2	20	15.0
3	20	25.1

induced the formation of a higher percentage of abnormal colonies (Table III). Cultures treated at a subtoxic dose of 1.2 $J \cdot m^{-2}$ exhibited no abnormal colony morphology and the treated cells would not grow in soft agar.

Tumor formation

The nodules which developed at the injection site in nude mice grew to be 0.6—1.05 cm in diameter and were well-encapsulated by mouse fibroblasts (Fig. 4). Removal of the tumors followed by karyological examination [1] confirmed that they were of human origin. The centers of the nodules were necrotic with dense infiltration of neutrophils and occasionally contained areas with extensive cholesterol cleft formation. Blood vessels

TABLE III

COMPARISON OF TOXIC EFFECT OF UV_{254nm} IRRADIATION WITH FREQUENCY OF ABNORMAL COLONY FORMATION AND ANCHORAGE INDEPENDENT GROWTH

UV ($J \cdot m^{-2}$)[a]	% Inhibition of colony formation	Abnormal colony[b] morphology 10^3 cells	Grow[c] in spec. act.
1.2	0.0	0.0	0.0
5.0	0.25	2.1	$10^{-3.4}$
10.0	0.50	3.5	$10^{-3.1}$
13.0	0.60	0.5	10^{-5}
13.5	0.65	0.5	0.0
16.0	0.95	0.0	0.0

[a] The above exposures were administered at 1.2 $J \cdot m^{-2} \cdot sec^{-1}$.
[b] After exposure to UV at the conclusion of S the cells were seeded at 10^3 cells/25 cm^{-2} well and cloned [9]. At the end of 9 days they were fixed, stained and enumerated.
[c] After 20 PDL, 50 000 cells were seeded in 2 ml of 0.33% agar. The cultures were refed once a week for 17 days. At that time the colonies were counted and the frequency of colony growth in soft agar reported as the number of colonies containing 50 or more cells/10^3 cells seeded.

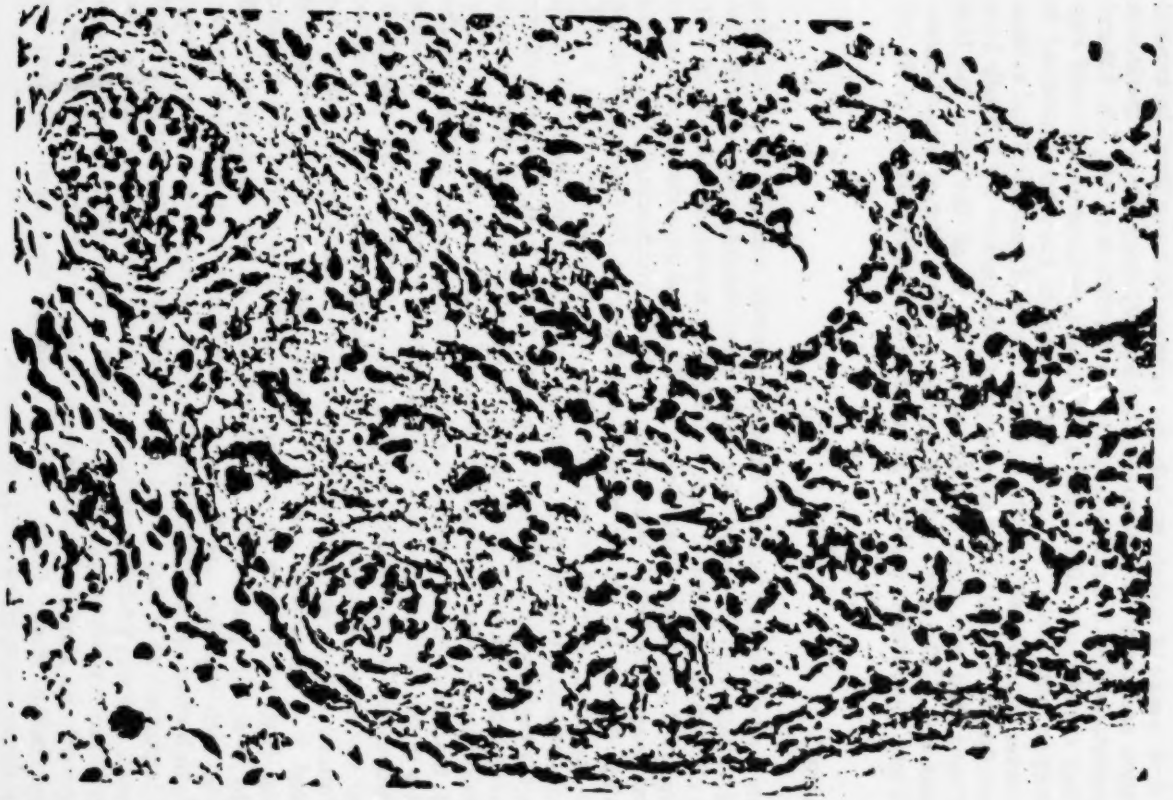

Fig. 4. Photomicrograph (120×) of a serial section of a tumor excised from the subscapular region of a nude mouse. The tumor was excised 4 weeks after injection of 5×10^4 cells. The tumor was fixed in buffered formalin and stained with hematoxylin and eosin.

were present within the nodules. The nodules were compatible with a benign proliferation of exogenous cells and could be described as myxofibroma-like.

Of 6 mice innoculated with the same inoculum, 4 developed tumors. After 4—6 weeks of nodule growth, the animals were sacrificed by cervical dislocation and the nodules removed for histopathology. These experiments were repeated 4X with frequencies of tumor formation of 4/6, 3/6, 5/6 and 3/6. They were fixed in 10% formalin, embedded, sectioned and stained with hematoxylin and eosin. To date, similar results have been obtained in 8 successive attempts while 200 irradiated untreated non-inoculated animals have not formed tumors. Fifty mice received a cell pack of 5×10^6 cells from 5 different lines and from 5 different tissues. In all the mice the bleb regressed within 72 h and the mice died 1—1.5 years later of natural causes. At autopsy there was no evidence of tumor at the site of injection of the cell pack.

DISCUSSION

Several parameters of putative changes in human foreskin cell populations during passage from the early stages of the transformation process to the late stages (growth in nude mice) were evaluated. Characteristics associated with UV-irradiated cell populations as early as PDL 5 and after discontinuation of the carcinogen treatment were altered colony morphology [1], altered saturation density [1] growth at 41°C and growth in 1% FBS-supplemented growth medium. Altered colony morphology was observed within 3 PDL after discontinued treatment. Immediately after the completion of *S* (8.2 h in length) the cells were split 1 : 10 and part of the treated cell population was cloned at 1000 cells · cm^{-2}. We observed colonies in the flasks or wells that exhibited a loss of contact inhibition and a criss-cross, disoriented piling up of the cells. These populations, when isolated from the rest of the colonies in the culture, gave rise to cell populations that exhibited abnormal morphology and an absence of the long parallel wholing growth patterns in normal untreated cultures. It was from these abnormal colonies that the transformed cell types (exhibit anchorage independent growth) arose. We observed 2—4 per 25-cm^{-2} well. In 8 treated wells we found 2 abnormal colonies that would grow in soft agar. Individual abnormal colonies also have to be serially passaged for 16—20 PDL before they would grow in soft agar. In each situation the frequency of growth in soft agar was similar; $10^{-3.5}$. In human cell transformation studies, abnormal colony morphology, while important as a tool to aid in determining if a transformation event happened, was not a reliable marker to quantitate the transformation events. In the refractory cell populations that would not exhibit anchorage independent growth, no abnormal colonies were observed during the early stages of the expression stage. These same phenomena have been observed for chemical carcinogen treated cell populations.

After 10 PDL, irradiated cell populations exhibited an alteration in lectin

agglutination profiles and grew at a temperature of 41°C. In fact, we found that 2—3 serial subpassages could be manipulated at 41°C. Normal cells will not survive 24 h at that temperature. However, at this point the cells still would not grow in soft agar, nor were they able to produce tumors in nude mice. Serial passage to 20 PDL was required to produce populations that would grow in soft agar.

After passage through soft agar and tumor formation in the nude mouse, UV-treated cell populations were able to grow to a finite PDL of 120—140. Cells with a normal phenotype phased out at PDL 40 ± 5. It is interesting to note that UV light has been shown to induce carcinogenesis in the skin of man although it has weak penetrating ability. UV light can be differentiated from X-ray by the specific nature of the induced damage and DNA repair processes. Owing to its particular features UV light, unlike many chemical carcinogens, does not require antecedent metabolic activation or specific binding before an interaction with cellular DNA.

The slopes of the toxicity profiles (survival curves) are similar to those seen for 10 T1/2 cells [17] on a per cell basis (Fig. 2). When we evaluated the survival response patterns of cells at risk 12 and/or 40 h after initiating treatment (4—8 h prior to the reinitiation of scheduled DNA synthesis), we found that by growing the cells at a low cell density the toxicity slopes (Fig. 3) were very similar to the slopes measured on a per cell basis (Fig. 2). We did note the presence of abnormal colonies and it was from these colonies that the neoplastically transformed cell populations arose. If we plotted the numbers of these abnormal colonies as a function of the dose in $J \cdot m^{-2}$ recorded as a frequency relative to the number of normal colony phenotypes, the optimum numbers of transformants occurred at a survival dose of 50 (ED_{50}) (Table III). At an ED_{65} the frequency of abnormal colonies was zero. The frequency of abnormal colonies we observed at ED_{25} was $10^{-3.6}$; at an ED_{50} it was $10^{-3.2}$; at an ED_{60} it was 10^{-5} (Table III). The same observations have been made for cell populations treated with chemical carcinogens of the type whose action does not require activation [1]. As in animal cell systems, treated human cells serially passage 24 h after exposure of the cells to UV, to give them time to replicate, enhances the formation of abnormal foci.

Therefore, in order to produce cell populations in the initial stages of a transformed phenotype the transformed cells must be allowed to replicate. When the treated cells were permitted to remain in a confluent density or non-proliferating phase of growth after treatment, no transformed cell populations were obtained. Contrary to the observation by Little [18] that survival is enhanced when mouse cells are left in growth-inhibited conditions after X-irradiation, human cells at that point are lost from the transformed phenotypic populations. However, the rate of repair processes in rodent cells is much slower than in human cells, which may explain why rodent cells must be allowed a longer repair time before they replicate and establish a transformed phenotype.

Since we can optimize the transformation events by irradiating NFS cells

in S, while treatment in G_1, or G_2 or M minimizes the events (Milo and DiPaolo, unpublished data), we believe that the process of UV-induced carcinogenesis in human fibroblast cells is complex, error prone and subject to critical timing of exposure to UV at an appropriate dose of the physical carcinogen. Following a selection process, the expression stage can be controlled by allowing at least 20 additional rounds of proliferation to occur before the cells will passage through soft agar and form tumors in the mouse.

In conclusion, we agree that the rate of recovery of cloning ability of human fibroblasts after UV treatment shows a correspondence with the ability of the cells to recover from the potential lethal effects of the irradiation [6]. However, we have found that even though we can demonstrate UV-induced transformation at toxic doses of ED_{50} or less, we cannot demonstrate transformation at higher toxic doses or lower non-toxic doses. Therefore, we feel that cell populations contain subsets of cells that are susceptible to carcinogen(s) but are lost from the total cell populations by modulation of the normal phenotype upon prolonged exposure to the tissue culture environment.

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